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# Disease-Corrected Hepatocyte-Like Cells from Familial Hypercholesterolemia-Induced Pluripotent Stem Cells

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**Abstract** The generation of human induced pluripotent stem cells (hiPSCs) from an individual patient provides a unique tool for disease modeling, drug discovery, and cell replacement therapies. Patient-specific pluripotent stem cells can be expanded in vitro and are thus suitable for genetic manipulations. To date, several genetic liver disorders have been modeled using patient-specific hiPSCs. Here, we present the generation of corrected hepatocyte-like cells (HLCs) from hiPSCs of a familial hypercholesterolemia (FH) patient with a homozygous mutation in the low-density lipoprotein receptor (*LDLR*) gene. We generated hiPSCs from a patient with FH with the mutated gene encoding a truncated non-functional receptor. In order to deliver normal *LDLR* to the defective cells, we used a plasmid vector carrying the normal receptor ORF to genetically transform the hiPSCs. The transformed cells were expanded and directed toward HLCs. Undifferentiated defective hiPSCs and HLCs differentiated from the defective hiPSCs did not have the ability to uptake labeled low-density lipoprotein (LDL) particles. The differentiated transformed hiPSCs showed LDL-uptake ability and the correction of disease phenotype as well as expressions of hepatocyte-specific markers. The functionality of differentiated cells was also confirmed by indo-cyanine green (ICG) uptake assay, PAS staining, inducible cyp450 activity, and oil red staining. These data suggest that hiPSC technology can be used for generation of disease-corrected, patient-specific HLCs with potential value for disease modeling and drug discovery as well as cell therapy applications in future.

**Keywords** *LDLR* gene therapy · Disease-specific human induced pluripotent stem cells · Hepatocyte differentiation · Familial hypercholesterolemia

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## Introduction

Familial hypercholesterolemia (FH) is primarily caused by inherited mutations in the low-density lipoprotein receptor (LDLR) encoding gene and is characterized by elevated blood cholesterol level. The deposition of cholesterol can result in xanthoma in the tendons of feet and hands, cutaneous planar, corneal arcus, and atheroma of the aortic root and valves, which can lead to fatal myocardial infarctions

before the age of 30 years [1–3]. Currently, FH management primarily involves lifestyle modifications and drug (statins) treatments [3, 4], so having a disease model based on iPSC technology in vitro might facilitate the process of finding new treatments and studying the impacts of specific allelic variations on control of low-density lipoprotein cholesterol. Recently, patient-specific iPSC-derived hepatocytes are shown to be a valuable tool for studying the functional contribution of various genomic loci in regulating lipid metabolism and recapitulating the complex pathophysiology of FH in culture [5].

Orthotopic liver transplantation (OLT) in homozygous patients is the best way to correct the disease phenotype [6], but this aggressive procedure carries considerable risks. Therefore, there is a need for development of alternative treatments. An ideal treatment for FH requires the genetic correction of patients' liver cells in order to restore normal phenotype. Successful ex vivo gene delivery experiments on rabbits and non-human primates [6–8] have demonstrated the feasibility of gene therapy for FH, which was subsequently performed in humans [9, 10]. In these first clinical experiments [9, 10], patients' primary hepatocytes were manipulated in vitro with retroviral vectors that carried the normal LDLR encoding gene and then injected back into the same patients. These treatments resulted in a stable therapeutic decline in blood LDL/HDL ratio. However, the invasiveness of the surgical intervention necessary for obtaining patients' hepatocytes and difficulties in culturing and maintenance of primary hepatocytes during the genetic manipulation procedure were the major drawbacks of this treatment protocol. In order to develop alternative therapies, we need to achieve a comprehensive insight about FH by in vitro evaluation of potential cell sources applicable for ex vivo genetic manipulation.

The generation of human induced pluripotent stem cells (hiPSCs) from dermal fibroblasts by epigenetic reprogramming [11] holds great promise for advancements in regenerative medicine and disease modeling. iPSCs can be generated from most mammalian somatic tissues [12] using different approaches [13] and can be differentiated into functional specialized cell lineages of all three embryonic germ layers. This wide differentiation potential provides fascinating possibilities and tools for drug discovery, developmental and pathogenesis studies, and investigation of possible treatment for inherited disorders (reviewed in [14]). To date, several diseases have been modeled [15–25] or corrected in vitro [26–29] using patients' derived iPSCs. A number of disease-specific cell lines have been generated from patients with genetic liver disorders such as α1 antitrypsin deficiency, glycogen storage disease types 1a and 1b, Crigler–Najjar syndrome, tyrosinemia type 1, progressive familial hereditary cholestasis, and FH [30, 31]. These

liver disease-specific iPSC lines have been differentiated toward hepatocyte-like cells (HLCs) and some of them were used for disease modeling; however, in none of the above cases genetic manipulation was examined as a means for correction of the disease phenotype. For the first time in this study, we generated disease-corrected HLCs from manipulated hiPSCs that were derived from the dermal fibroblasts of a homozygous FH patient.

## Materials and Methods

### Generation of hiPSCs from Patient's Fibroblasts

Dermal fibroblasts were harvested from a 14-year-old female patient with homozygous FH [32]. The ethical principles of the Helsinki Declaration were followed and the human dermal biopsy sampling was approved by the local Royan Institutional Review Board and Ethical Committee. There were nine independent patient-specific hiPSC clones (FH-hiPSCs) generated from the patient's dermal fibroblasts by transduction of retroviral vectors carrying OCT4, SOX2, c-MYC, and KLF4, as described earlier in serum and feeder-free culture conditions [33].

### Karyotype Analysis and Bisulfite Sequencing

Karyotype analysis and bisulfite sequencing were performed as described previously [34]. The promoter regions of human OCT4 and NANOG gene were amplified with PCR and the products were subcloned into the InsTAclone PCR Cloning Kit (Fermentas). The cloned fragments were sequenced with M13 universal primers and analyzed with BIQ Analyzer software.

### RT-PCR and Quantitative RT-PCR Analysis

Total RNA was isolated using the RNeasy Kit (Fermentas). Two micrograms of total RNA was used for reverse transcription reaction with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and random hexamer primer (Fermentas), according to the manufacturer's instructions. The first strand cDNA was used for PCR reaction and the products were examined by electrophoresis on 2–3 % agarose gel in TAE buffer.

Quantitative real-time PCR (qRT-PCR) reactions were set up in experimental duplicate for the three biological repeats with the Power SYBR Green Master Mix (Applied Biosystems) and analyzed with a 7500 RT-PCR system (Applied Biosystems). Expression values were normalized to the average expression of housekeeping gene [ActB by the comparative CT method $(2^{-\Delta\Delta c_t})$]. The primer sequences have been previously presented [31, 33].

## Genetic Manipulation of FH-hiPSCs

The pENTR/D-TOPO and pLenti6/UbC/V5-DEST vectors (Invitrogen, K2400-20 and V499-10) were used for cloning *LDLR* cDNA (Variant number 1, accession number: NM_000527.4). The pLenti6/UbC/V5-DEST derived construct that carried the normal ORF was subsequently used for lipofection of FH-hiPSCs with Fugene6 reagent (Roche, 05061377001). Transfection was performed according to the manufacturer's instructions, 24 h after plating 200,000 single cells per well in a 6-well plate.

## Direct Differentiation of hiPSCs into HLCs

Human induced pluripotent stem cells differentiation toward the hepatic lineage was performed using Basma et al. [35] protocol with some modifications. Briefly, embryoid bodies (EBs) were generated by plating collagenase/dispase-treated cells at a density of $1–5 \times 10^4$ cells/cm$^2$ on bacterial petri dishes for 48 h in DMEM/F12 supplemented with 20 % KOSR, 1 mM nonessential amino acids, and 2 mM L-glutamine. The EBs were then plated on Matrigel-coated plates in DMEM/F12 supplemented with activin A (100 ng/mL, R&D, 338-AC) for 3 days to induce a definitive endoderm lineage. The concentration of KOSR was started from 0 % for the first 24 h, changed to 0.2 % for the second 24 h and 2.0 % for the final 24 h. Cells were then grown for 8 days in DMEM/F12 that contained 2.0 % KOSR, 1 mM nonessential amino acids, 2 mM L-glutamine, 1 % dimethyl sulfoxide (Sigma-Aldrich), and 100 ng/mL HGF (R&D Systems, 294-HG). The culture was continued for an additional 5 days in hepatocyte culture medium (HCM) (Lonza, Inc., CC-1399) that consisted of 2 % KOSR, 1 mM nonessential amino acids, 2 mM L-glutamine, and 0.1 μM dexamethasone (Sigma-Aldrich, D-2915).

## Alkaline Phosphatase Staining, Immunofluorescence Staining, and Flow Cytometry

Alkaline phosphatase (ALP) staining was conducted based on the manufacturer's recommendations (Sigma, 86R).

Immunofluorescence staining for pluripotency and hepatic markers in addition to flow cytometry analysis for ALB and asialoglycoprotein receptor 1 (ASGPR1) were performed as described previously [31, 34].

## Low-Density Lipoprotein (LDL) Uptake Assay

LDL Dylight™ 549 kit was used (Cayman Chemical, 10011125) and the assay was performed according to the manufacturer's instructions. Cells were visualized using a fluorescence microscope (IX71, Olympus, Japan).

## Cytochrome P450 Activity and Inducibility

Cytochrome P450-dependent pentoxyresorufin *O*-dealkylase activity (PROD) was evaluated using pentoxyresorufin substrates. Pentoxyresorufin is O-dealkylated by CYP and changes a nonfluorescent compound into resorufin, a fluorescent compound [36]. To evaluate the inducibility of cytochrome P450, the differentiated cells were exposed to sodium phenobarbital for three days and subsequently washed. Then an incubation mixture containing 7-pentoxyresorufin substrate (Sigma-Aldrich) and dicumarol (Sigma-Aldrich) in Hank's balanced salt solution (HBSS) was added and plates were incubated at 37 °C in a 5 % $CO_2$ incubator for 30 min. Cell supernatant medium was collected after 30 min for three biological repeats and examined for the quantity of resorufin produced using a fluorospectrophotometer (Cary-Eclipse, Varian) at 530 nm excitation/590 nm emission against resorufin standards. To give a comparative view, the results were normalized to the number of cells.

## Periodic Acid-Schiff (PAS) Staining

Glycogen storage of hiPSC-derived HLCs was evaluated by PAS staining at day 23. The cells were fixed with 4 % paraformaldehyde, oxidized in 1 % periodic acid for 5 min, then washed and treated with Schiff's reagent for 15 min, with subsequent color development in dH$_2$O for 5–10 min and assessed under light microscope (BX71, Olympus).

## ICG Uptake and Release

The day 23 differentiated cells were incubated with ICG (Cardiogreen, Sigma-Aldrich) in basal medium for 1 h at 37 °C. Uptake of ICG was detected by light microscopy (IX51, Olympus). ICG elimination from the positive cells was verified after 6 h.

## Secretion Analysis

On day 23, conditioned media with fully differentiated hiPSCs were collected and stored at −20 °C until assayed. The media were assayed for alpha-fetoprotein (AFP) secretion using a chemiluminescence immunoassay kit (Pishtaz-Teb); for ALB secretion with an ALB ELISA kit (Bethyl); and for urea secretion with a colorimetric assay kit (Pars Azmun) according to the manufacturers' instructions. Secretion was normalized to the total number of cells.

## Oil Red Staining

The storage of lipid vesicles in cells was assessed by oil red staining. Differentiated cells were fixed with 4 % paraformaldehyde, and incubated for 1 h with oil red. The stained cells were washed and analyzed with an inverted light microscope (IX51, Olympus).

## Statistical Analysis

mRNA expression levels and other experiments were measured for three independent biological repeats and expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the Student's $t$ test for independent samples. The mean difference was considered significant at the $p < 0.05$ level.

## Results

### Generation and Characterization of FH-hiPSCs

Dermal fibroblasts were harvested from the skin biopsy of a 14-year-old homozygous FH female patient. The patient had tendon xanthoma, xanthelasma, and a total blood cholesterol level of 801 mg/dL. The LDLR encoding gene of the harvested cells was previously shown to have a single base pair insertion in position 2411 in exon 17 [32]. The abnormal phenotype is the result of this frame-shift in the *LDLR* coding sequence and the subsequent production of a truncated non-functional receptor. Next, these somatic cells were reprogrammed to iPSCs under feeder and serum-free conditions by the 4-factor approach as described before [33]. We obtained nine hiPSC colonies (FH-hiPSC3) out of around 200,000 plated human fibroblast cells. Of these, one hiPSC colony was picked and expanded for further characterization. The cell line had the normal karyotype (Fig. 1a) and was used for further analyses. The cell line exhibited the characteristic human embryonic stem cell (hESC) morphology (colonies of round compact cells with high nucleus to cytoplasm ratio) and strong ALP activity. FH-hiPSCs also expressed pluripotency markers including OCT4, SSEA4, TRA-1-60, and TRA-1-81 (Fig. 1a). The pluripotency of the FH-hiPSCs was further assessed by RT-PCR analysis of lineage specific markers after spontaneous differentiation (Fig. 1b). The methylation status of the *OCT4* and *NANOG* promoters was also monitored with bisulfite sequencing, which revealed that these regions were highly unmethylated in hESCs and established hiPSCs, whereas the CpG dinucleotides of the regions were highly methylated in the parental HDFs (Fig. 1c). The qRT-PCR analysis, with primers specific for retroviral transcripts of *OCT4*, *SOX2*, *c-MYC*, and *KLK4*, indicated complete silencing of the four

transgenes (data not shown). The line maintained a normal karyotype in a continuous culture for several months by weekly passaging with a split ratio of 1:3 to 1:6. Collectively, these results demonstrated that the hiPSC line established in this study was fully reprogrammed.

### Genetic Transformation of FH-hiPSCs

A normal human LDLR complete ORF was PCR amplified from the normal HDFs' cDNA and cloned into the pLenti6/UbC/V5-DEST vector under the control of the ubiquitin (UbC) constitutive promoter. DNA sequencing revealed the proper cloning of the LDLR ORF in the vector, without any mutations. Next, the plasmid was used to transform feeder-free cultured FH-hiPSCs. Blasticidin (10 µg/mL) was added into medium from day 3. This dose of Blasticidin was observed to kill all non-transfected FH-hiPSCs in 3 days. After 3 weeks, a single genetic-transformed FH-hiPSC (GT-FH-hiPSC) colony was generated from the starting population of around 1,200,000 iPSCs. The resistant colony was picked up, expanded and differentiated toward hepatocytes according to the protocol described previously [35].

### Generation of Disease-Free HLCs

Morphological analysis of the cells revealed that during the differentiation process, the hiPSCs gradually formed polygonal and highly granulated HLCs with less density, and a lower nucleus to cytoplasm ratio (Fig. 2a). Functional correction of the GT-FH-HLCs was demonstrated by LDL-uptake assays (Fig. 2a). Immunofluorescence staining of LDLR in HLCs using a polyclonal antibody that binds both normal LDLR and the truncated non-functional receptor of the defective cells demonstrated the expression of this receptor in the cells (Fig. 2a). However, the defective FH-HLCs did not show the ability to uptake fluorescent LDL particles from the medium and this function was restored in GT-FH-HLCs. The morphology, receptor staining, and LDL-uptake of HLCs derived from hESCs (Royan H6) [37] were used as positive controls for the experiment. RT-PCR expression analysis of hiPSCs as well as FH-hiPSC- and GT-FH-hiPSC-derived HLCs (FH-HLCs and GT-FH-HLCs), with primers specific for the exogenous LDLR encoding mRNA, also revealed expression of exogenous receptor mRNA only in the GT cell lines (Fig. 2b). qRT-PCR expression analysis of the endogenous LDLR mRNA showed up-regulation of the receptor in FH-hiPSCs and its down-regulation in GT-FH-HLCs upon differentiation (Fig. 2c).

To further assess the transition from hiPSCs to HLCs in direct differentiation, qRT-PCR analysis was performed on hiPSCs and hiPSC-HLCs for expression of hepatic lineage cells (*AFP*, *ALB*, *HNF4α*, and *CYP3A4*) and the undifferentiated hiPSCs marker (*OCT4*). The results showed that
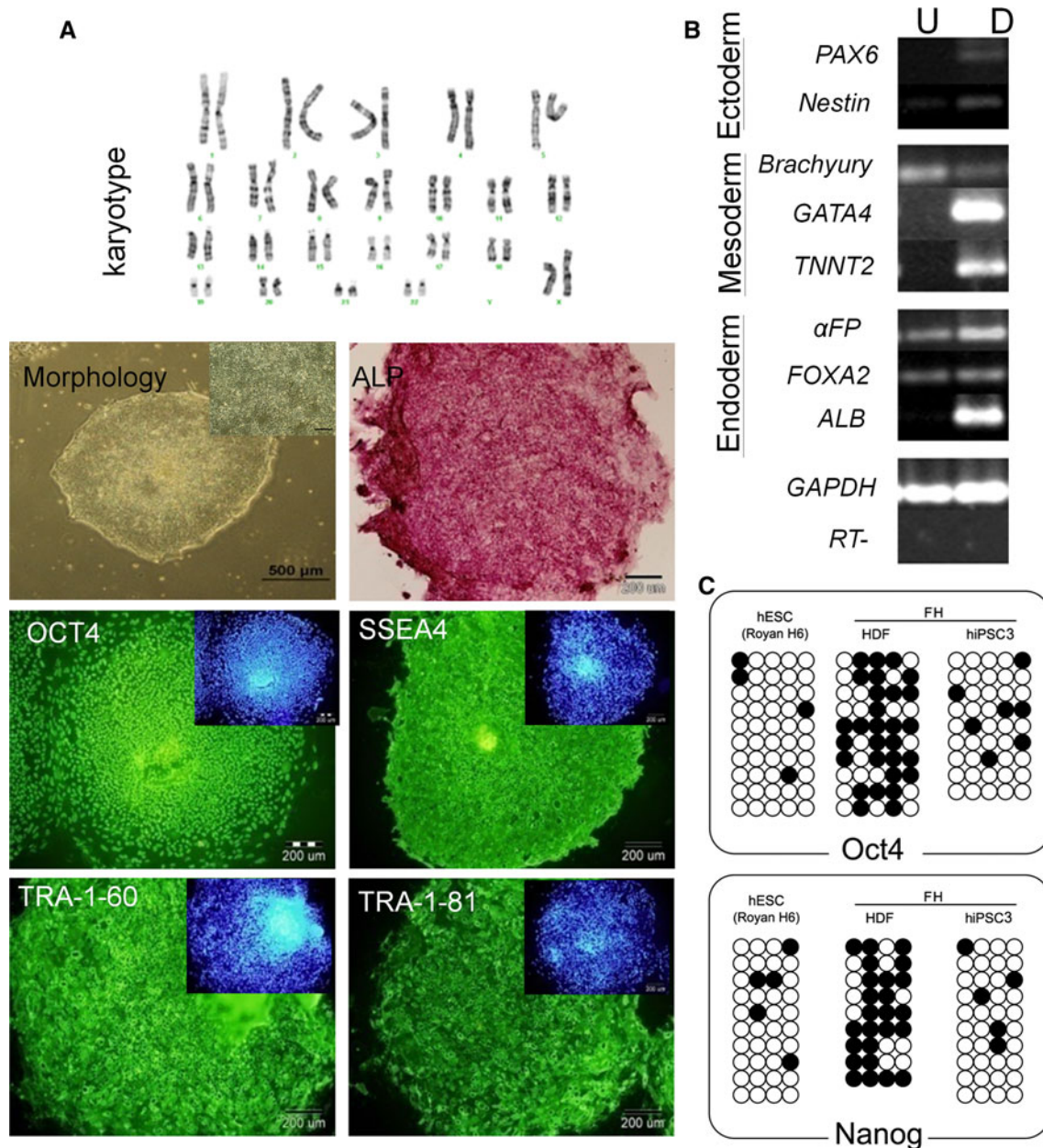
**Fig. 1** Generation of iPSCs from patient's fibroblasts. **a** Normal karyotype, morphology, and expression of different pluripotency and surface markers of FH-hiPSC cell line are presented. The lines are characterized after at least ten passages. Nuclei were stained with DAPI (blue). **b** RT-PCR analyses of various differentiation markers for the three germ layers in spontaneously differentiated "D" and undifferentiated "U" cells. **c** Bisulfite genomic sequencing of the promoter regions of *OCT4* and *NANOG*. *Open* and *closed circles* indicate unmethylated and methylated CpGs, respectively. Human ESCs (*Royan H6*) is the control (Color figure online)

while *OCT4* mRNA level decreased significantly upon hepatic induction; HLCs-specific mRNAs were increased (Fig. 3a). The expression differences between FH-HLCs and GT-FH-HLCs were not statistically significant (Fig. 3a). Immunofluorescence staining for ALB, AAT, and CYP1A1 verified hepatic-specific gene expression at the protein level (Fig. 3b). Flow cytometry was performed to determine the percentage of ALB and ASGPR1 expression, which is a definitive feature of hepatocytes.

The percent of positive cells in the populations of FH-HLCs and GT-FH-HLCs were 22 and 25 % for ASGPR1 and 61 and 62 % for ALB, respectively (Fig. 3c).

To evaluate the other functionalities of FH-HLCs and GT-FH-HLCs, cells were examined for hepatic-specific functions in vitro. The cells' ability to store lipids was demonstrated by oil red staining (Fig. 4a) and their glycogen storage ability was proved by PAS staining (Fig. 4b) and the presence of specific membrane transporters by ICG
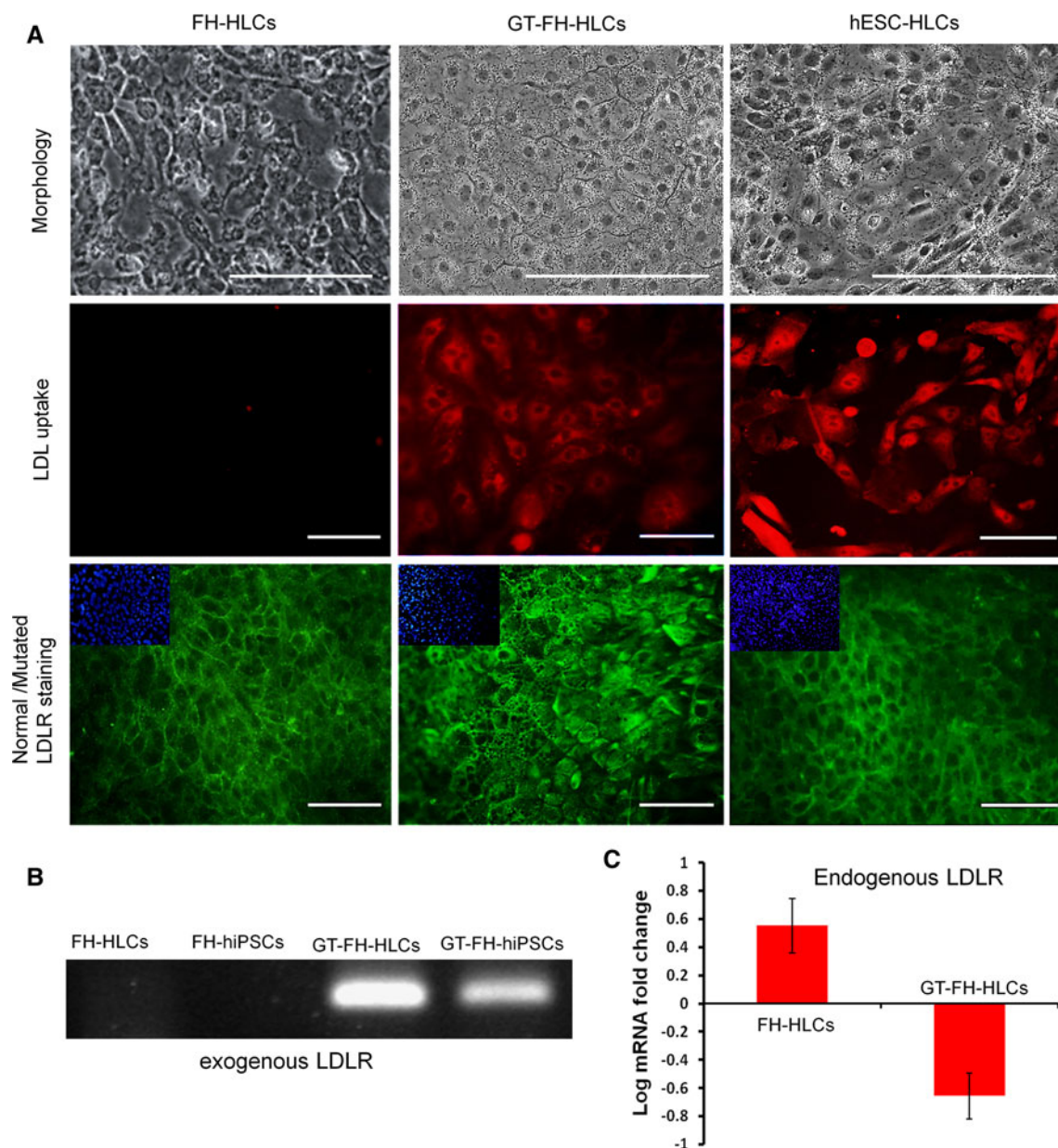
**Fig. 2** Functional correction in GT-FH-HLCs. **a** FH-HLCs and GT-FH-HLCs were shown to have polygonal and granulated morphological characteristics of mature hepatocytes. LDLR staining with a polyclonal antibody that binds both normal and mutated receptors showed the presence of LDLR on FH-HLCs and GT-FH-HLCs membrane. However, the LDLRs of the FH-HLCs were not functional and unable to uptake LDL particles. The LDL-uptake assay showed the successful gain of function upon genetic transformation of FH-hiPSCs and their differentiation. hESC (Royan H6)-derived HLCs were used as positive controls. Nuclei were stained with DAPI (blue). *Scale bar* 100 μm. **b** Exogenous *LDLR* gene was only expressed in genetic-transformed cells. **c** Endogenous *LDLR* gene was down-regulated in HLCs derived from genetic-transformed cells relative to those derived from defective cells (Color figure online)

uptake (Fig. 4c). Hepatocytes-like cells had also high secretion activity and were able to secret ALB, AFP, and urea to the medium (Fig. 4d–f). The PROD test was performed to assess human hepatocyte-specific cytochrome P450 activity before and after induction with phenobarbital and the results showed an approximate 1.5-fold increased activity of CYP upon induction ($p < 0.05$, Student's $t$ test, Fig. 4g). All of the above assays were performed for three biological repeats and the differences between FH-HLCs and GT-FH-HLCs were not statistically significant.

## Discussion

Hepatocytes are responsible for about 70 % of body LDL-uptake. Genetic defects that interfere with this hepatic
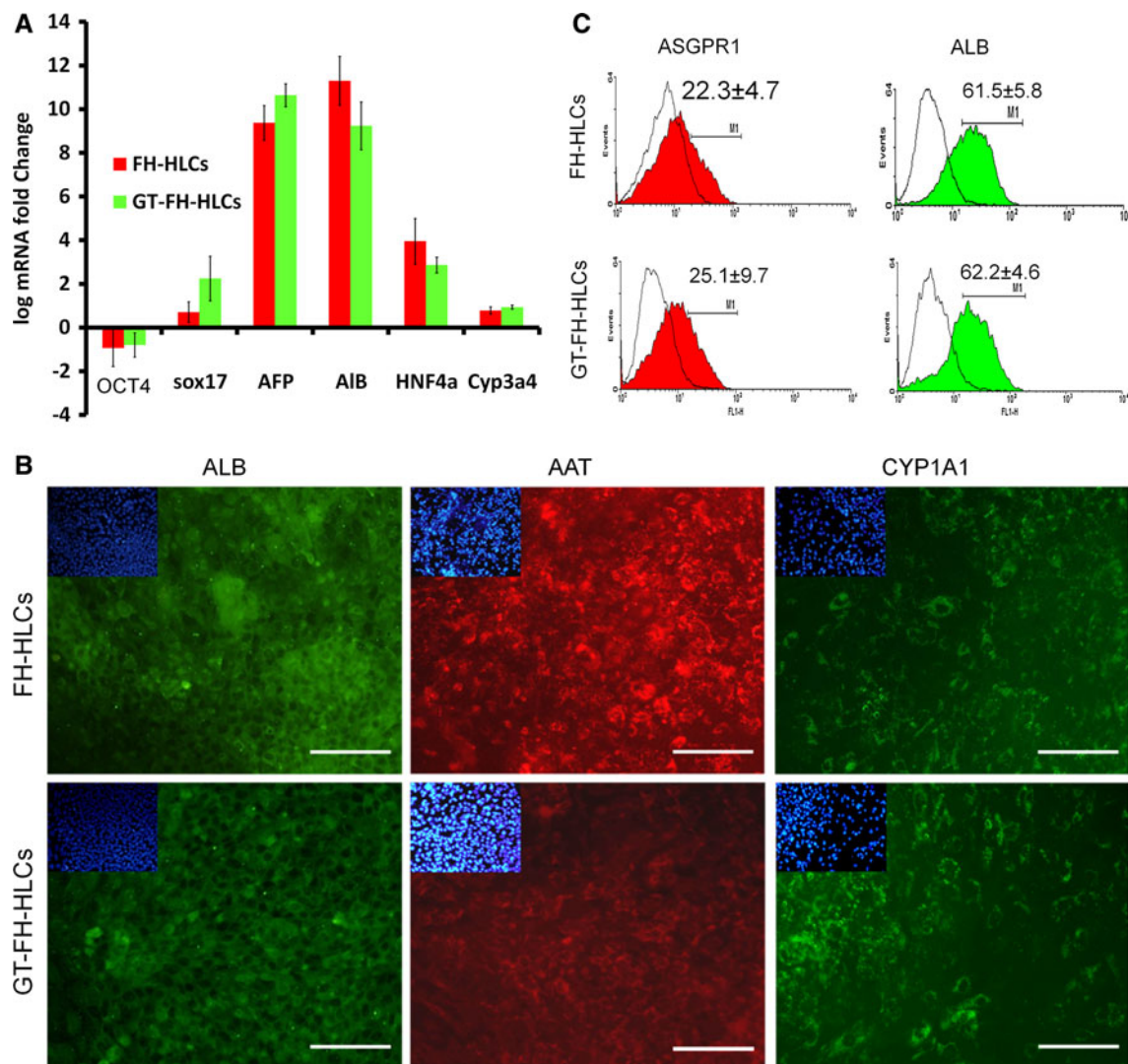
**Fig. 3** mRNA and protein expression in differentiated cells. Directed differentiation of hiPSCs toward HLCs, and characterization of FH-HLCs and GT-FH-HLCs. **a** Gene expression analysis. The mRNA fold change of hepatic-specific genes (*AFP*, *ALB*, *HNF4A*, and *CYP3A4*) and a pluripotency gene (*OCT4*) have been examined for corrected and non-corrected hiPSC–HLCs relative to their undifferentiated stage. Expressions of endoderm and hepatocyte markers increased during the time span of differentiation whereas *OCT4* expression decreased. **b** Protein expression analysis by immunofluorescence staining. HLCs were positive for hepatocyte-specific markers (ALB, AAT, and CYP1A) at the end of the differentiation period. The data proves efficient induction and maturation of FH-HLCS and GT-FH-HLCs. Nuclei were stained with DAPI (blue). *Scale bar* 100 μm. **c** Efficient differentiation was further confirmed by flow cytometry quantification of ALB and ASGPR1 positive cells (Color figure online)

function will lead to the lethal disorder of FH. To date, OLT has been the only available option for curing this severe genetic liver disorder. Due to the major limitations of OLT (e.g., the availability of suitable donors and the risk of an immune response), the search for a replacement therapy has been an attractive issue for many scientists over the past decade [9, 10, 38].

In this study, we represent the differentiation of hiPSCs into HLCs expressed various liver markers and liver-specific functions, such as albumin secretion, urea production, ICG glycogen storage, and inducible cytochrome P450 activity. Considering the gene expression and functional analysis, these cells showed characteristics of immature fetal hepatocytes as reported previously [39, 40].

Further, we have previously showed that patient-specific hiPSCs can be generated in serum and feeder-free culture conditions from patients suffering from various genetic liver disorders [31]. Several recent papers have reported that regardless of the origin of the reprogrammed somatic cell, the generated hiPSCs can be directed to hepatocytes in the same manner as hESCs [30, 31, 41–44]. These cell lines can serve as tools for modeling disorders, drug screening, and therapeutic applications [45–47].
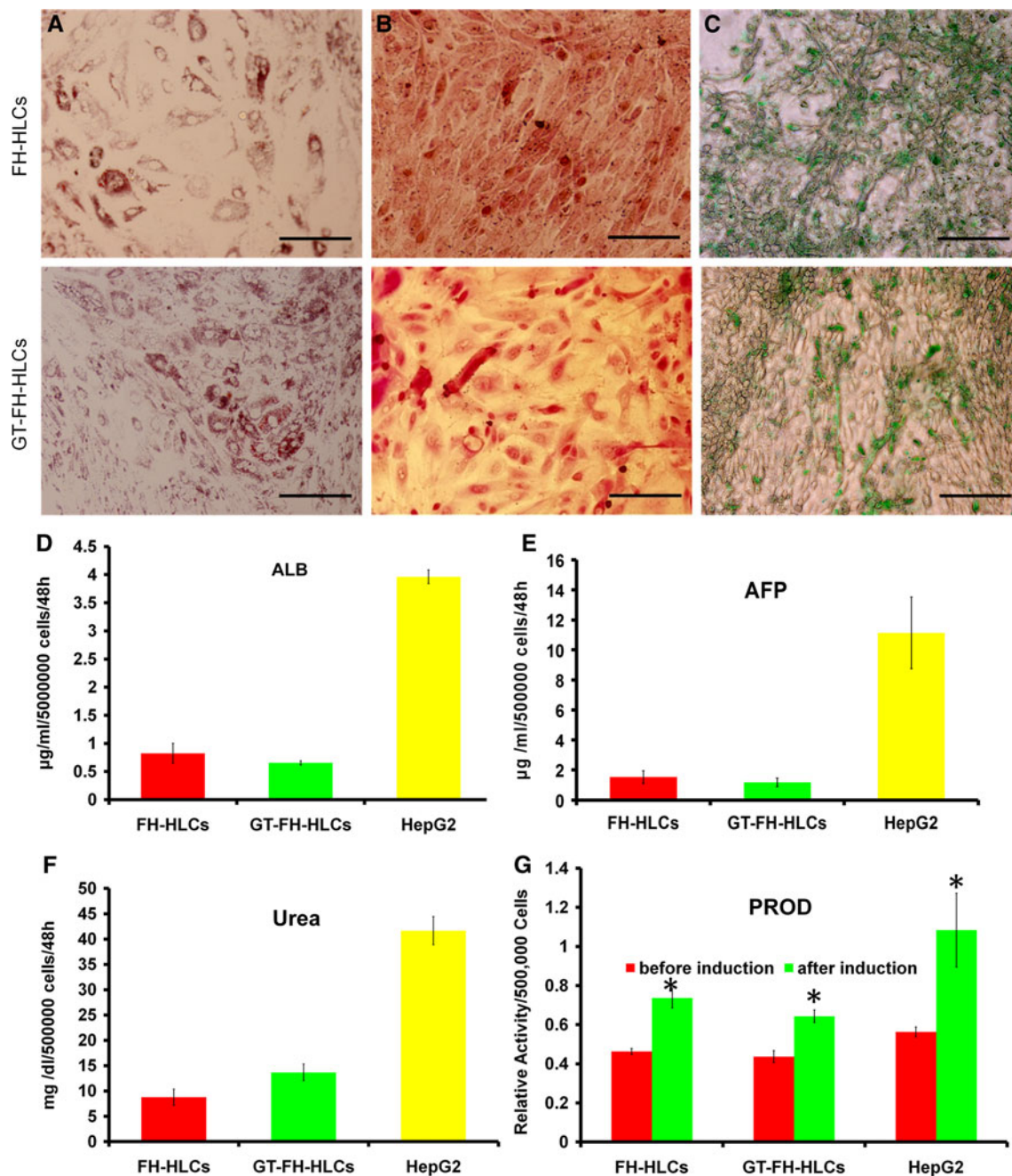
**Fig. 4** Hepatocyte-specific functional assays. Qualitative functions of the differentiated cells derived from both cell lines were examined. Cells were able to **a** store lipid (oil red staining) and **b** glycogen, as well as (**c**) uptake anionic ICG from culture media. *Scale bar* 100 μm. Quantitative functions of the differentiated cells from both cell lines were examined. **d** ALB, **e** AFP, and **f** urea secretion during the time span of differentiation indicate the functionality of the cells in vitro. **g** Cyp450 activity and its inducibility upon phenobarbital adminis-tration. PROD activity increased significantly in response to pheno-barbital induction ($p < 0.05$, Student's $t$ test). The differences between FH-HLCs and GT-FH-HLCs were not statistically significant (Color figure online)

We observed that the hiPSCs–HLCs had hepatic-spe-cific functions in vitro; however, due to a mutation in the LDL receptor gene which is responsible for most of the liver LDL-uptake, differentiated cells from the defective patient-specific iPSCs were not able to uptake LDL. On the other hand, the genetically transformed hiPSC–HLCs were

shown to uptake LDL from the media, which has proven successful return of the lost function. Exogenous *LDLR* mRNA was expressed under control of a housekeeping promoter (UbC) in genetically transformed cells. This promoter expresses the receptor in physiological amounts and the presence of LDL in differentiating media results in
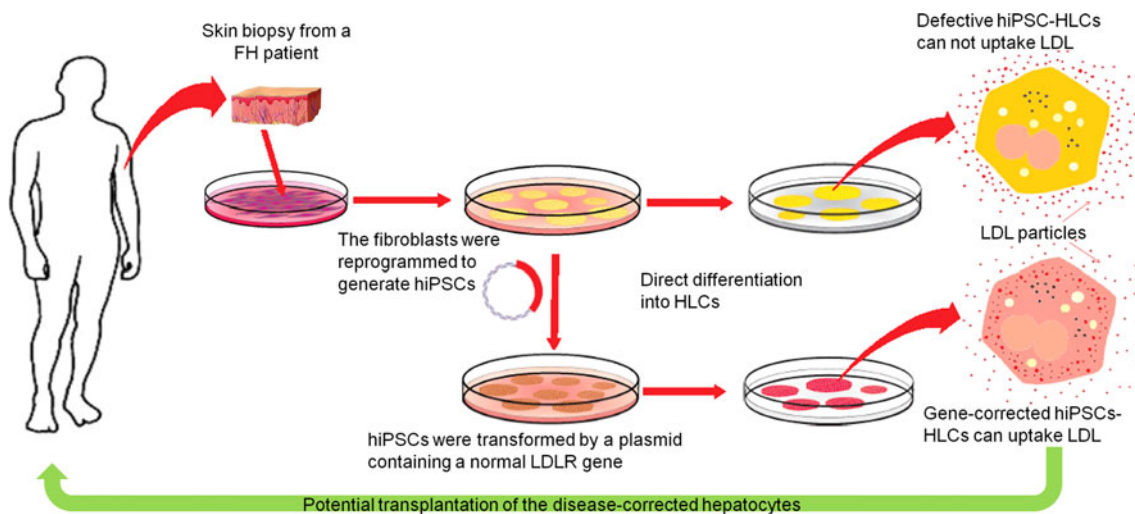
**Fig. 5** Patient-specific disease-free hepatocytes generated from familial hypercholesterolemia-induced pluripotent stem cells (Color figure online)

the in vitro incorporation of these particles in functionally corrected cells. This incorporation activates the negative feed-back loop [48, 49] in GT-FH-HLCs and results in a decrease in endogenous *LDLR* mRNA.

These disease-corrected HLCs from patient-specific hiPSCs might provide an accessible therapeutic source (Fig. 5), as the principle for therapeutic application of iPSC-HLCs has been proved by treating diseases in animal models [50]. It has been reported that iPSC-HLCs have both functional and proliferative potential for liver regeneration after transplantation in an acute liver failure model or after partial hepatectomy in mice with fumarylacetoacetate hydrolase deficiency [51] as well as their potential as a source for disease modeling and evaluation of the pathophysiology of the metabolic diseases such as FH [5]. Therefore, the next step to evaluate the therapeutic potential of GT-hiPSC–HLCs is to examine them in vivo in animal models of FH, such as *LDLR*-knockout mice [52] and Watanabe heritable hyperlipidemic (WHHL) rabbits [53].

Taken together, our results suggest the possible application of iPS technology and gene correction methods for personalized medicine and bring new hopes for treatment of metabolic liver patients. However, before clinical application of these genetic manipulated hiPSC–HLCs, more detailed studies are needed to address safety issues concerning iPSCs production [54], immunologic response [55], tumor formation [56], and genetic manipulations. In addition, further studies are required to show the functionality of cell in vivo. There is also a need for development of more efficient, chemically defined differentiation protocols. Recently, it has been shown that functional HLCs can also be generated directly from mouse fibroblasts [57]. This strategy can be an alternative way for producing disease-specific HLCs applicable for genetic modifications and gene therapy. Further studies, however, are needed to examine this approach on human fibroblasts. Until then, FH-HLCs and GT-FH-HLCs can serve as tools for the basic investigation of the LDL-uptake pathway and in vitro disease models for studying FH and its potential treatments.

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